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TEST OF A MOSQUITO EGGSHELL ISOLATION METHOD AND SUBSAMPLING PROCEDURE

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ABSTRACT. Production of *Aedes vigilax*, the common salt-marsh mosquito, can be assessed by determining eggshell densities found in soil. In this study, 14 field-collected eggshell samples were used to test a subsampling technique and compare eggshell counts obtained with a flotation method to those obtained by direct examination of sediment (DES). Relative precision of the subsampling technique was assessed by determining the minimum number of subsamples required to estimate the true mean and confidence interval of a sample at a predetermined confidence level. A regression line was fitted to cube-root transformed eggshell counts obtained from flotation and DES and found to be significant ($P < 0.001$, $r^2 = 0.97$). The flotation method allowed processing of samples in about one-third of the time required by DES, but recovered an average of 44% of the eggshells present. Eggshells obtained with the flotation method can be used to predict those from DES using the following equation: DES count = $[1.386 \times (\text{flotation count})^{0.33} - 0.01]^3$.

INTRODUCTION

The most abundant estuarine wetland mosquito found along the east coast of Australia is *Aedes vigilax* (Skuse), an important vector of Ross River virus in humans and heartworm in dogs (Russell 1993). One approach to the study of *Ae. vigilax* and other mosquito populations involves counting eggshells found in sediments (Kay and Jorgensen 1986; Ritchie and Addison 1991, 1992; Ritchie and Johnson 1991; Ritchie 1994; Ritchie and Jennings 1994). Because the spatial distribution of mosquito eggshells is generally stable, locations favorable for oviposition and hatching may bear large numbers of eggshells (Ritchie 1994). Eggshell sampling may be useful for the investigation of mosquito management options and assessment of wetland rehabilitation (Ritchie 1994, Ritchie and Jennings 1994, Streever 1997). However, eggshell sampling requires a separation technique to allow cost-effective enumeration of eggshells.

A recently developed method for the extraction of eggshells from wetland soil involves a flotation process in which eggshells are separated from higher density particles in a water column. The portion of a sediment sample that floats is examined under a dissecting microscope for eggshells (Ritchie and Addison 1991, 1992; Ritchie 1994; Ritchie and Jennings 1994). A fundamental difference between this and the flotation technique originally described by Horsfall (1956) is that whole eggs were separated from debris in a saturated NaCl solution in the earlier study. Some researchers have estimated recovery rates for the more recent method by adding a known number of eggshells to sand and clay substrates (Ritchie and Jennings 1994) or eggshell-free mangrove soil (Ritchie and Addison 1991) and then counting the number of eggshells recovered following flotation. However, variable recovery rates may result from differences in bulk density, consistency, fibrous root content, and detritus content of soil collected from wetland sites. Thus, recovery rates estimated by adding known numbers of eggshells to specific soil types may not be representative of the wide range of soils found at some wetland sites.

In this study, field-collected eggshell samples were used to test a subsampling technique and compare eggshell counts obtained with a flotation method to those obtained by direct examination of sediment (DES).

MATERIALS AND METHODS

Sediment samples were collected from the salt marsh bordering 4 tidal creeks on the southern side of Kooragang Island, New South Wales, Australia, about 120 km north of Sydney. Dominant halophytes included *Sarcocornia quinqueflora* (Bunge *ex* Ungen-Sternberg) (Chenopodiaceae) and *Sporobolus virginicus* (Linn.) Kunth (Poaceae), with *Triglochin striata* Ruiz and Pavon (Juncaginaceae), and *Suaeda australis* (R. Br.) Moq. (Chenopodiaceae) occurring as subdominants (Buckney 1987). Sixteen soil cores were collected from a 1-m^2 area at each of 14 sampling stations. Each soil core was taken with a plastic corer (2.9-cm diam) to a depth of about 2.2 cm, giving a soil volume of 15 cm^3. The 16 cores collected at each sampling station were pooled to give a total soil volume of approximately 240 cm^3 per sample. Each sample was added to 250 ml of water, shaken vigorously for 1 min, and then washed through 3 sieves (300-, 250-, and 150-μm mesh aperture) to isolate eggshells and similar-sized particles. The 150-μm sieve isolates eggshells, which are known to range between 188.6 and 217.1 μm in diameter at their widest point (Linley et al. 1992). Occasionally, small quantities of root-bound soil did not break apart and would not pass through the top sieve. This material was dispersed by blending for 10 sec and subsequently passed through the sieves.

Twenty subsamples were extracted from each of the 14 sieved samples. Material from the 150-μm

sieve was poured into a 2-liter beaker and topped up to 200 ml with tap water. A 2-ml glass pipette was used to agitate the solution and keep the soil in suspension. Twenty 2-ml subsamples were withdrawn from the suspension. Thus, the total subsample volume was 40 ml, or about one-fifth of the total volume. Each subsample was examined under a stereomicroscope and the numbers of hatched eggshells, unhatched eggshells, and unhatched eggs were recorded.

An eggshell was defined as hatched if it had a distinct line of dehiscence. Unhatched eggshells were usually flattened and discolored without any hatching lines. Eggs were defined as whole and cylindrical. As a major emphasis of eggshell sampling is the identification of sites suitable for larval production, only hatched eggshells were considered in any analysis. Eggshell fragments and opercula were not counted. All sediment, eggs, and eggshells were placed back into the sample after all 20 subsamples had been processed.

The following equation was used to determine subsampling precision (Southwood 1978):

$$n = (st_{\alpha}/Em)^2,$$

where n is the required subsample size, m is the mean of the 20 subsamples, s is the standard deviation for the 20 subsamples, E is the half-width of the confidence interval as a decimal of the mean, and t_{α} is from the Student's t-distribution. We set E at 10% of the mean and adjusted α to 3 levels: 0.10, 0.05, and 0.01. Thus, the equation gives an estimated sample size (n) allowing 90, 95, or 99% confidence that the true mean is within 10% of the estimated mean. Estimated sample size was corrected using the finite population correction (FPC) because the subsampling procedure removed a significant proportion (20%) of the total sample (Krebs 1989). The formula is:

$$n^* = n/[1 + (n - 1)/N],$$

where n^* equals the corrected subsample value, n is calculated from Southwood's formula, and N equals the maximum number of subsamples (100). This correction limits the value of n to a maximum of 100, equating to the entire sample. Standard errors quoted for mean hatched eggshell count per subsample were also modified using FPC.

The flotation procedure was carried out on the same 14 samples to provide a 2nd estimate of hatched eggshell density. For each sample, 40 ml of material was taken from the 200-ml suspension using the subsampling technique. Samples were air-dried on the 150-μm sieve for at least 24 h. The dried material was brushed from the sieve into a Petri dish where it was broken down with the fingers. The powdered material was placed back into the sieve, wetted, and washed from the sieve into a 500-ml beaker filled with water. The beaker was placed into the sieve and overtopped with a fine jet of water. Eggshells and other light material within the beaker overflowed into the sieve. Water and suspended material remaining in the beaker was poured into the sieve after about 60–120 sec of applying the water to the sample. Dense particles remained in the bottom of the beaker. Material trapped within the sieve was examined for eggs and eggshells and their numbers were recorded.

Table 1. Mean *Aedes vigilax* hatched eggshell count, confidence intervals, and the required subsample size to be 90, 95, and 99% confident that the true mean is within 10% of the estimated mean.

Sample	Mean number of eggshells per subsample	Lower and upper 95% confidence limits for the mean[1]	Required subsample size 90% CI[2]	95% CI	99% CI
1	0.05	0.00–0.14	98	99	99
2	0.15	0.00–0.30	95	96	98
3	1.35	0.77–1.93	76	82	90
4	1.90	1.39–2.41	55	64	77
5	3.10	2.42–3.78	45	55	69
6	3.90	3.22–4.58	34	43	59
7	5.40	4.48–6.32	34	43	58
8	9.30	7.93–10.67	27	35	50
9	13.15	11.93–14.37	13	18	29
10	27.95	26.11–29.79	7	10	17
11	28.25	25.52–30.98	14	19	31
12	49.75	45.44–54.06	11	16	26
13	172.4	161.01–183.79	7	10	17
14	477.5	439.75–515.25	10	14	23

[1] Based on corrected SE for a finite population.
[2] CI = confidence interval.

A Wilcoxon's matched-pairs test was used to compare the recovery of hatched eggshells with DES and flotation, as well as the time required (min) to carry out both procedures. For flotation, time included the flotation procedure except for drying time. Sorting times for both methods included the allocation of eggshells to broad color categories. However, eggshell color is not considered in this paper. Regression analysis was used to determine the relationship between hatched eggshell counts from the DES and flotation methods. The DES and flotation counts were cube-root transformed to stabilize variances before regression analysis (Emerson and Stoto 1983).

RESULTS AND DISCUSSION

The mean subsample count (hatched eggshells) for the 14 samples ranged from 0.05 (SE = 0.04, n = 20) to 477.5 (SE = 18.04, n = 20). Table 1 shows the subsample mean, 95% confidence limits, and required subsample size to attain a predetermined level of precision for each sample. Subsample requirements increased with decreasing mean eggshell count; however, 95% confidence intervals are relatively narrow. Thus, the use of 20 subsamples per sample gave a good indication of the true eggshell density.

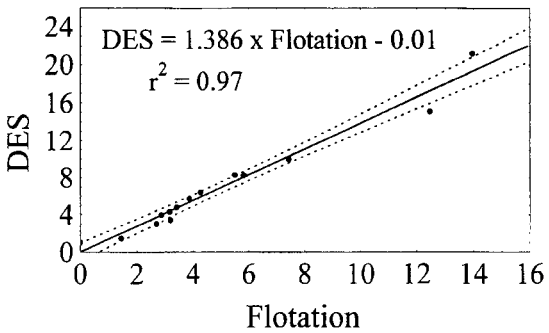

Fig. 1. The relationship between *Aedes vigilax* eggshell counts for the flotation method and the direct examination of sediment (DES). Counts were cube-root transformed. Dashed lines represent 95% confidence bands for the regression line.

The mean number of hatched eggshells per sample using DES was 1,134.6 (SE = 692.2, n = 14), whereas the flotation method yielded 407.3 (SE = 223.3, n = 14). The DES counts were significantly greater than those recorded with flotation (Wilcoxon's matched-pairs test, Z = 3.2, $P < 0.002$, n = 14). For each sample, it took an average of about 3 times longer to process eggshells with DES than with flotation; each sample required a mean of 278 min (SE = 63, n = 14) to process with DES and 92 min (SE = 19, n = 14) to process with the flotation method. This difference was significant (Wilcoxon's matched-pairs test, Z = 3.3, $P < 0.001$, n = 14). Although the flotation method was more rapid than DES, fewer eggshells were recovered with the flotation procedure. Estimated mean eggshell recovery using flotation was 44% (SE = 6.8, n = 14). Ritchie and Jennings (1994) recovered between 30 and 71.7% of *Ae. vigilax* eggshells from spiked soil cores. Recovery depended on the technique used to disperse the soil, soil type (sand and clay), and eggshell color. Ritchie and Addison (1991) reported a recovery rate varying between 41 and 62% for spiked *Aedes taeniorhynchus* (Wied.) eggs and eggshells but could not demonstrate any effect of eggshell color on recovery.

A significant relationship (regression analysis, $P < 0.001$, n = 14) exists between the number of hatched *Ae. vigilax* eggshells recovered using DES and the number recovered using the flotation method (Fig. 1). The large coefficient of determination (r^2 = 0.97) suggests that little information is lost when the flotation method is used to estimate DES counts using the formula: DES count = $[1.386 \times (\text{flotation count})^{0.33} - 0.01)^3$, which is the back-transformed regression equation of Fig. 1. Thus, counts obtained from the less labor-intensive flotation method can be easily converted to estimate DES counts. The strong fit of the regression line suggests that the effect of subsampling variability is minimal; poor or inadequate subsampling would have reduced the r^2 value.

In summary, little information was lost when subsampling and flotation were used to reduce soil processing and eggshell enumeration time. Subsampling one-fifth of a sample is adequate with the soil volume and technique employed. However, the number of subsamples will be largely dictated by the nature of the field study, as a greater number of subsamples will improve the efficiency of the flotation method at low eggshell densities.

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